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TITLE: TGF-Beta Gene Polymorphisms in Food Allergic versus Non-Food Allergic Eosinophilic Esophagitis

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14. ABSTRACT

The diagnosis of eosinophilic esophagitis (EoE) is based on the presence of $\geq$ 15 eosinophils/hpf in the esophagus of a patient with symptoms of esophageal dysfunction in whom GERD is excluded. EoE is likely mediated by interaction of environmental allergens (such as foods) with several genes. Food antigens play an essential role in EoE since specific food elimination diets and amino acid formulas are successful EoE therapy in 60-98% of subjects. Indeed, the majority of children with EoE have specific IgE to foods but they often continue to ingest these foods due to lack of immediate hypersensitivity reactions. This study focuses on the gene-environment interaction of food consumption in food sensitized children with EoE and TGFb1 gene polymorphisms. We hypothesize that in EoE there is a gene polymorphism (TGFb1) environment (food) interaction that contributes to increased IgE mediated TGFb1 expression in the esophagus and increased esophageal remodeling in a subset of EoE subjects. As esophageal stricture formation is an important complication of remodeling in EoE (6-12% of children; 33% of adults), identifying genetic polymorphisms in TGFb1 in EoE may allow the early identification of food sensitized children at risk for the development of this significant complication of EoE.

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Table of Contents

Introduction:
The diagnosis of eosinophilic esophagitis (EoE) is based on the presence of ≥ 15 eosinophils/hpf in the esophagus of a patient with symptoms of esophageal dysfunction (i.e. dysphagia, anorexia, early satiety, failure to thrive) in whom gastro-esophageal reflux disease has been ruled out by lack of response to treatment with a proton pump inhibitor. The prevalence of EoE is approximately 1:2,500 in pediatric populations the focus of this study. Although there is evidence of a familial association of EoE, EoE as with other food allergic and allergic diseases is likely mediated by interaction of environmental allergens (such as foods) with several genes. Food antigens play an essential role in EoE since specific food elimination diets and amino acid formulas are successful as EoE therapy in 60-98% of subjects. Indeed, the majority of children with EoE have specific IgE to foods but they often continue to ingest these foods due to lack of immediate hypersensitivity reactions. This study focuses on the gene-environment interaction of food consumption in food sensitized children with EoE and the role of TGFb1 gene polymorphisms. We hypothesize that in EoE there is a gene polymorphism (TGFb1) environment (food) interaction that contributes to increased IgE mediated TGFb1 expression in the esophagus and increased esophageal remodeling in a subset of EoE subjects. As esophageal stricture formation is an important complication of remodeling in EoE (6-12% of children; 33% of adults), identifying genetic polymorphisms in TGFb1 in EoE may allow the early identification of food sensitized children at risk for the development of this significant complication of EoE. The TGF-b1 gene promoter has a well characterized functional SNP C-509T which results in three genotypes, i.e. TT, CT, or CC. Prior studies have demonstrated that the TT genotype of the TGF-b1 gene is associated with increased levels of fibrosis in progressive kidney disease. We will examine whether the TT genotype is associated with increased fibrosis in food sensitized EE subjects. We hypothesize that the TGF-b TT genotype is associated with increased levels of TGF-b expression in the esophagus in food sensitized EE and promotes the development of esophageal remodeling.

Body:
This proposal outlines 6 tasks to be completed during the three year proposal. Task 1 (approval of human subjects) was completed in year 1 of this proposal. In current year 2 we have continued to work on tasks 2-5 (proposed to be completed in year 3 by month 33) as outlined in our original proposal. Task 6 (manuscript submission) is planned for year 3 (months 33-36).

Task 1: Approval for human subjects studies (month 1-6)
UCSD IRB approval of the DOD modified consents was obtained on April-5-2011.
We received an approval letter from DOD permitting us to start working on the project on April 13, 2011 in an e-mail from Ms Duchesneau (Chief, Human Subjects Protection Review; Caryn.Duchesneau@us.army.mil).

Task 2: Enrolling EE (Food IgE+ and Food IgE-) subjects (n= 400 subjects) (month 1-33)
2a) Database for EE genotyping clinical trial established (month 1)
There have been 74 new EE subjects enrolled in current year 2 (September 2012 through August 2013), which exceeds the annual goal of 60 new patients. Combining this with our previously accumulated patient population brings the total number of EoE subjects in the database to 461. We have now exceeded the minimum number of 400 subjects needed in the database to carry out the proposed study. We have genotyped 152 subjects whose information is entered into our database on an ongoing basis (see below). We are currently in the process of assessing those subjects who are food IgE+ versus food IgE negative. Analysis of genotype in the context of food allergy phenotype is underway.
2b) Demographic and clinical information entered (month 1-33)
Demographic and clinical information for all new subjects (n=74) have been entered into the database on a weekly basis. Similar data is available from previously entered subjects. The current analysis shows that, consistent with past reports, the majority of our EE subjects are male, Caucasian, and have another atopic disorder (asthma, allergy, eczema and/or food allergy) (n=142, analysis is ongoing) (**Tables 1, 2**). Overall, approximately 61% of EE subjects are Food IgE+ and 39% are Food IgE- **(Table 1).** In the Food IgE+ group 30-40% have significant positive IgE to milk, wheat, egg, and/or soy (**Table 3**) with food specific serum IgE elevated in the range of 2-4 kU/L (normal < 0.35) among those subjects who have had serum IgE testing completed. (**Tables 4**).

Table 1: **Demographic and Food Allergic Characteristics of Pediatric EoE Population**

Age in years (range)	Male (%)	Ethnicity, (n=142)	Food IgE* Positive	Food IgE Negative
7 (6.2-7.9)	82	Hispanic- 12 Non-Hispanic- 130 American Indian/Alaskan Native-0 Asian- 5 Black- 7 Native Hawaiian, Other Pacific Islander- 1 Other/Mixed Race- 6 White- 111	61%	39%

*Serum or skin prick testing positive

Table 2: Co-existent Allergic Characteristics of Pediatric EoE Population

Asthma (%)	Allergic Rhinitis (%)	Eczema (%)
43%	66%	30%

Table 3: Food IgE Sensitization Pattern

Food Antigen	IgE Positive (% patients, 95% CI) (n=122-129)
Egg	39% (30, 48)
Milk	39% (30, 47)
Wheat	35% (26, 43)
Soy	33% (24, 41)

*IgE positive is defined as food serum IgE >0.35, or food skin prick test wheal/flare >3/5mm as compared to negative saline control

Table 4: Levels of serum IgE to Foods

Serum IgE, mean ku/L			
Egg (n=69)	Milk (n=70)	Wheat (n=69)	Soy (n=68)
3.4	2.4	3.3	3.4

Normal IgE < 0.35; n= number of study subjects with positive serum IgE

2c) Results of upper GI endoscopy entered (month 1-33)

Upper GI endoscopy results have been entered on a weekly basis for all new subjects (n=74)

2d) Results of esophageal biopsy eosinophils/hpf entered (month 1-33)

Pathology reports are generated in 2 days following the endoscopy with biopsy and results are entered into the database on a weekly ongoing basis (see graphs below for distribution by genotype).

<u>Task 3:</u> Quantitating esophageal expression of TGF-b and pSmad in EE (Food IgE+ and Food IgE-) (n=400 subjects)(month 1-33)

3a) Making pathology blocks of esophageal biopsy (month 1-33)

Esophageal biopsy blocks are made following each endoscopy and biopsy (n= 74)

3b) Sectioning esophageal biopsy blocks (month 1-33)

Sectioning of esophageal biopsy blocks is ongoing on a bi-weekly basis (n=74). Sections are first evaluated for the presence of lamina propria (LP) and those with LP are preferentially evaluated.

3c) TGF-b immunostain: To quantitate TGF-b+ cells (month 1-33)

Quantitation of TGFb+ cells: Immunohistochemistry and quantitation is ongoing. To date 89 EoE biopsies with adequate lamina have been evaluated for TGFb+ cells. Preliminary data reinforces the hypothesis that there are more TGFb1 expressing cells in TGF-b genotype TT subjects as compared with either CC or CT subjects ($p<0.001$ TT versus CT; $p<0.001$ TT versus CC) (Fig 1). With the additional subjects from this past year, we have found that CT subjects also have significantly more TGFb+ cells than CC subjects ($p<0.05$) (Fig 1). The process of analyzing subject phenotype for food allergy versus non-allergic in the context genotype has begun and will continue until month 33.

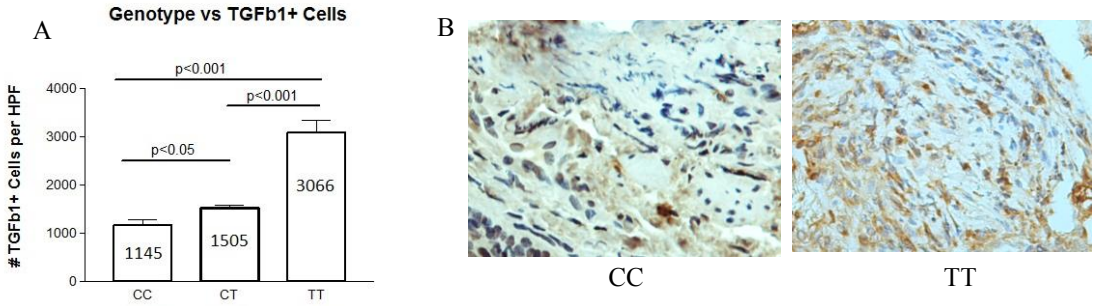

Figure 1. Subjects with TT TGF beta genotype have significantly higher numbers of TGFb1 positive cells as compared with CC or CT genotype subjects (A). A representative image (B) of immunohistochemistry for TGFb1 in a CC and a TT subject

3d) pSmad immunostain : To quantitate pSmad+ cells (month 1-33)

Immunohistochemistry and quantitation is ongoing. To date 79 EoE biopsies with adequate lamina propria have been evaluated for pSmad+ cells. These data do not show elevated levels of pSmad+ cells by genotype to date.

3e) MBP Ab; Immunostain to quantitate eosinophils (month 1-33)

Evaluation of MBP has been done on a subset of EoE biopsies. The numbers of MBP positive cells correlates well with the numbers of eosinophils seen on H&E ($r=0.81$, $p<0.0001$) and as such H&E is a complementary method for the numbers of MBP positive cells. A representative image of MBP staining using immunofluorescence is shown below (**Fig 2A)** as is the relationship of epithelial and LP eosinophils to TGF beta genotype (n=108 EE subjects for Epithelium, n=72 EE subjects for LP) which shows a trend for increased LP eosinophils in TT TGF beta genotype patients but no clear correlation between epithelial eosinophils and genotype.

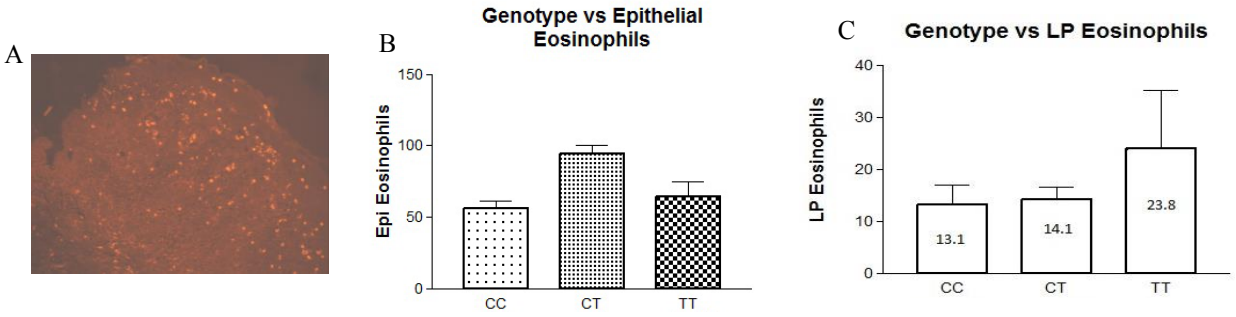

Figure 2. A representative image of MBP positive eosinophils in the epithelium of an EoE subject (A). Quantitation of epithelial eosinophils (B) and LP eosinophils (C) shows a trend towards increased eosinophils in the LP of TT TGF beta genotype subjects.

3f) Control Abs (month 1-33)

Control Abs are routinely used to immunostain slides to exclude non-specific staining of tissues. We have not noted any non-specific staining with either the control Abs for TGF-b1 Ab or pSmad.

3g) Slides (month 1-33)

Esophageal tissue sections are sectioned onto slides on a routine basis.

3h) Results of esophageal biopsy histology (task 3a-g) entered into the database.

Esophageal histology results are entered into the database on a weekly basis.

<u>Task 4:</u> TGF-b genotyping in EE (month 1-33)

4a) Consent for genotyping in EE subjects (month 1-33)

EoE subjects are consented on an ongoing basis for genotyping. To date 37 new EoE subjects have been consented for genotyping.

4b) TGF-b genotyping (month 1-33)

Genotyping is ongoing and occurs in batches. We have updated the methods for genotyping subjects to utilize SNP specific primers and taqman based PCR. A subset of these samples are to be sent for sequencing in order to verify accuracy. This is a significant improvement to the SNP analysis as it is faster than the prior restriction digest based assay. Since September 2012, 37 additional subjects have been consented for genotyping. To date 152 EoE subjects have been genotyped.

4c) TGF-b single nucleotide polymorphisms (SNP) information entered into database (month 1-33)

SNP information is entered into the database on an ongoing basis

4d) Results of TGF-b SNP genotyping, phenotyping, and TGF-b/pSmad analysed

Analysis is ongoing and larger numbers are needed to make definitive conclusions regarding genotype and phenotype. The data analysis to date demonstrates that TGFb1 positive cells are significantly higher in TT TGF beta genotype subjects as compared with CC or CT TGF beta genotype subjects (n=74) (Figure 1).

<u>Task 5:</u> Quantitating esophageal remodeling in EE (Food IgE+ and Food IgE-) (month 28-33)

5a) Trichrome stain: To quantitate fibrosis

A subset of biopsy specimens with adequate LP have been assessed for trichrome staining and analysis to see if the trichrome stain fibrosis score correlates with that seen on H&E. We have found that fibrosis scoring using H&E is accurate as compared with trichrome, requires less tissue by decreasing the numbers of paraffin cuts, and we have utilized this methodology for fibrosis scores. Note that this task was not planned to begin until year 3 (month 28-33), but has started early in year 2 in months 12-24. There is a trend towards TT TGF beta genotype subjects having higher fibrosis scores than CC or CT subjects. We hypothesize that we may be able to detect some statistical significance as the numbers increase (Figure 3).

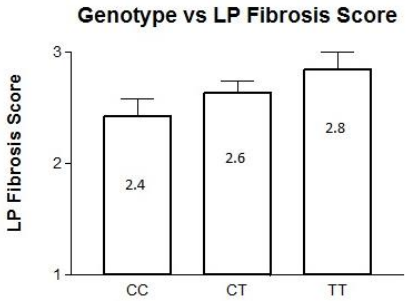

Figure 3. Subjects with TT TGF beta genotype have a statistically insignificant trend to higher lamina propria (LP) fibrosis scores as compared with CC or CT genotype subjects. There is also a statistically insignificant trend for a stepwise increase in the fibrosis score from CC to CT to TT

5b) H and E stain: To quantitate basal zone hyperplasia (month 28-33)

Quantitation of basal zone hyperplasia, epithelial score, and the LP score have been completed in 108 subjects for epithelium and in 72 subjects for LP. Preliminary data demonstrates a trend toward more severe fibrosis and LP eosinophilia in TT subjects. Note that this task was not planned to begin until year 3 (month 28-33).

5c) VWF Immunostain: To quantitate blood vessels (month 28-33)

We have stained for VWF in 65 subjects. Note that this task was not planned to begin until year 3 in month 28. To date in year 2 there is a trend towards higher blood vessel numbers in the CT and TT TGF-b genotype subjects that may reach statistical significance with higher subject numbers (Figure 4).

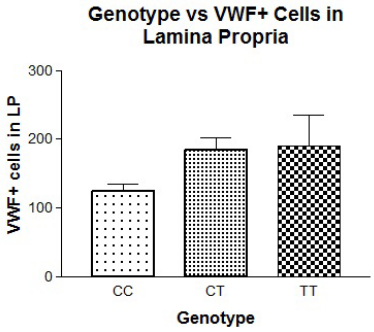

Figure 4. Subjects with CT and TT TGF-beta genotype have a statistically insignificant trend to higher numbers of blood vessels using a VWF antibody to stain vascular endothelium.

5d) VCAM Immunostain: To quantitate activation of blood vessels (month 28-33)

VCAM staining will begin shortly. Note that this task was not planned to begin until year 3 month 28.

5e) Control abs (month 28-33)

Control Abs are used to immunostain slides to exclude non-specific staining of tissues.

5f) Slides (month 28-33)

Esophageal tissue sections are sectioned onto slides on a routine basis

<u>Task 6:</u> Preparation of manuscript (month 33-36)

We plan to prepare a manuscript in year 3 by month 33.

We have presented an abstract of our preliminary findings at the AAAAI meeting (2013) in San Antonio (see below).

Key Research Accomplishments:

- Enrollment of 74 new EoE subjects (results in 461 total EoE subjects in the database)
- Enrollment of 37 additional EoE subjects for genetic studies
- Genotyping of 152 EoE subjects to date
- Analysis of TGFb1 and pSMAD immunostaining in 89 and 79 subjects, respectively
- Analysis of vWF staining in 64 subjects

Reportable Outcomes:

Preliminary results reported in abstract form (see below) suggest that the functional C-509T SNP in the TGFb1 gene may influence EoE histology and phenotype. Final reportable outcome of this observation in original manuscript form will require completion of the analysis of additional subjects in year 3 through month 33 as originally proposed.

Conclusion:

Preliminary conclusions as above.

References:

None.

Appendices:

Abstract 475 presented by by Anilkumar AA et al at AAAAI (2013) attached.

Manuscripts/Reprints, Abstracts:

Abstract:

Anilkumar AA, Newbury RO, Dohil R, Mueller J, Hoffman HM, **Broide DH, Aceves SS.** A Transforming Growth Factor Beta-1 Gene Single Nucleotide Polymorphism May Influence Phenotype in Pediatric Eosinophilic Esophagitis. *J Allergy Clin Immunol*;131:S132. 2013. Presented as an oral abstract in at AAAAI meeting in March 2013

A transforming growth factor beta-1 gene single nucleotide polymorphism may influence phenotype in pediatric eosinophilic esophagitis

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Rationale:
Eosinophilic esophagitis (EoE) is a chronic antigen driven disease associated with tissue remodeling and increased TGFb1 expression. The genetic influences on disease severity and therapeutic response are currently unknown. A functional single nucleotide polymorphism (SNP), C-509T, in the TGFβ1 gene promoter has been linked to asthma and renal disease severity. We hypothesized that this SNP may influence histologic and/or phenotypic findings in pediatric EoE.

Methods:
We performed single nucleotide polymorphism analysis for TGFβ1 C-509T in pediatric EoE subjects. Histology scores were generated by a pathologist blinded to genotype and clinical course. TGFb1+ cells were quantitated using immunohistochemistry and image analysis. Subject response to topical corticosteroids was evaluated in the context of genotype.

Results:
Of 129 EoE subjects, 41 (32%), 75 (58%), and 13 (10%) were genotype CC, CT, and TT, respectively. 63 subjects had adequate lamina propria (LP) for analysis. Subjects with genotype TT had significantly more TGFβ+ cells (mean=2511cells/mm^2, SEM =150) than genotypes CT (1532cells/mm^2, SEM=64) or CC (mean=997cells/mm^2, SEM=90) ($p < .0001$). TT subjects had higher numbers of LP eosinophils (20/ hpf versus CT-13/hpf and CC -12/hpf) while CC subjects had lower numbers of peak epithelial eosinophils (mean=57.9 per hpf) than CT (100.2/hpf) or TT (66.7/hpf) subjects ($p<0.001$). 49 subjects were treated with topical esophageal corticosteroids (TCS). While 94% of CC subjects responded to TCS, CT and TT subjects had more variable response rates of 73% and 57%, respectively (OR=4.6, p=0.08).

Conclusion: The functional C-509T SNP in the TGFβ1 gene may influence EoE histology and phenotype.

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